Permeable guidance channels containing microfilament scaffolds enhance axon growth and maturation

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Abstract: Successful peripheral nerve regeneration is still limited in artificial conduits, especially for long lesion gaps. In this study, porous poly(L-lactide-*co*-DL-lactide, 75:25) (PLA) conduits were manufactured with 16 poly(L-lactide) (PLLA) microfilaments aligned inside the lumen. Fourteen and 18 mm lesion gaps were created in a rat sciatic nerve lesion model. To evaluate the combined effect of permeable PLA conduits and microfilament bundles on axon growth, four types of implants were tested for each lesion gap: PLA conduits with 16 filaments; PLA conduits without filaments; silicone conduits with 16 filaments; and silicone conduits without filaments. Ten weeks following implantation, regeneration within the distal nerve was compared between corresponding groups. Antibodies against the markers S100, calcitonin gene related peptide (CGRP), RMDO95, and P0 were used to identify Schwann cells, unmyelinated axons, myelinated axons, and myelin, respectively. Results demonstrated that the filament scaffold enhanced tissue cable formation and Schwann cell migration in all groups. The filament scaffold enhanced axonal regeneration toward the distal stump, especially across long lesion gaps, but significance was only achieved with PLA conduits. When compared to corresponding silicone conduits, permeable PLA conduits enhanced myelinated axon regeneration across both lesion gaps and achieved significance only in combination with filament scaffolds. Myelin staining indicated PLA conduits supported axon myelination with better myelin quantity and quality when compared to silicone conduits.
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Key words: nerve conduit; contact guidance; PLA; neural tissue engineering

INTRODUCTION

The success of peripheral nerve regeneration is still limited in artificial conduits, especially over long lesion gaps.[1] Most researchers have focused on conduits made of natural polymers, such as collagen,[2–7] fibronectin,[8,9] alginate,[10–12] agarose,[13,14]and hyaluronic acid.[15,16] As with tissue conduits (such as vein), these conduits are often unable to support growth across long lesion gaps due to the possibility of collapse, scar formation, and early resorption.[17] Natural polymers might also induce undesirable immune responses with additional disadvantages including batch-to-batch variation in large-scale isolation procedures. To avoid these problems, synthetic biomaterials are being widely explored for neural implantation. Silicone conduits have been tremendously useful for studying nerve regeneration. However, problems associated with chronic nerve compression have been confirmed with these biodurable conduits,[18,19] which require a subsequent reoperative removal of the conduit. Synthetic bioreabsorbable conduits hold the primary benefits of flexibility of chemical and mechanical characteristics, lack of antigenicity, ease of availability, and the avoidance of problems associated with chronic persistance. To promote long-term nerve recovery, conduits made of biodegradable materials, such as polylactide (PLA) or polyglycolide (PGA),[20–23] poly(lactide-*co*-caprolactone) (PLC),[24–27] poly(glycolide trimethylene carbonate) (GTMC),[28,29] poly(3-hydroxybutyrate) (PHB),[30] or polyphosphoesters[31,32] have attracted great attention.

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Regenerative failure is evident in simple silicone guidance channels above a critical gap length of about 10 mm in a rat sciatic nerve implantation model.[33] Considerable efforts to improve the regeneration environment inside the conduit, including adding neurotrophic factors,[34–39] extracellular matrix molecules,[8,40–42] and Schwann cells[20,21,43–48] have been applied. Still, the lack of inner guidance structure for enhancing nonneuronal cell migration across the lesion gap may limit nerve regeneration, especially over long lesion gaps.

One of the striking characters of autologous nerve grafts, the current "golden standard" treatment for peripheral nerve injury, was the endogenous longitudinal nerve structural alignment and cellular components of the normal nerve. Williams et al.[49] first acknowledged this longitudinal pattern as a "prerequisite" for nerve regeneration, in which he observed delayed nerve growth from randomly oriented fibrin strands. Earlier studies using denatured muscle autografts also demonstrated the impact of longitudinal organization for nerve regeneration. Muscle grafts take advantage of the similarities between tubular skeletal muscle basement membrane and endoneurial tubes of degenerating nerves. Vein-filled muscle grafts were claimed as the most encouraging clinical trials as an alternative nerve graft.[50] However, there is still some controversy concerning the balance of complete muscle proteins denaturing and the correct orientation of the retaining basal tubes.

The impact of longitudinal patterning for nerve regeneration is being gradually realized as an important factor in modern artificial conduit design for nerve injury repair. Miller[51,52] and Thompson[53] designed oriented Schwann cells and DRG neurite outgrowth on micropatterned polymer substrates *in vitro*. Magnetically aligned collagen gel inside collagen conduits and implanted them into 6-mm surgical gaps in mice sciatic nerves, yielding superior regeneration compared to nerve guides filled with control collagen gel.[3,4] Bundles of collagen filaments (20-μm diameter) were grafted to bridge a 30-mm defect of rat sciatic nerve, which reported high numbers of myelinated axon at the implant distal end 12 weeks postoperatively.[54] Longitudinally aligned collagen fibers supported nerve fiber regeneration as long as they stay intact; upon degradation nerve regeneration was hampered.[55] Lundborg[56] first reported a bioartificial "intrinsic framework" inside silicone conduits by eight nylon filaments in 10-mm rat sciatic nerve injury model. Later, Itoh et al.[57] reported a similar bridging graft (15 mm) made from silicone conduits containing eight collagen filaments or filaments of PLA and fatty acid copolymer. Except the unpleasant large diameter of polymer filaments (150–200 μm), the above-mentioned two reports described no details concerning the ability of the filaments as contact guidance for axon growth.

Previous data from our laboratory have demonstrated that poly(L-lactide) (PLLA) microfilaments serve as structural support for nerve regeneration, especially across long lesion gaps. *In vitro*, Schwann cells were found to grow on the microfilaments, where neurites from dorsal root ganglia extended and oriented longitudinally along them.[58] This may be due to the inherent stiffness of the cytoskeletal structure of axons being resistant to bending forces so that preferred axon growth is along the path of minimal principle curvature, or the long axis of the filaments (Dr. Patrick Tresco, personal communication). Thus, the guidance characteristics are highly dependent on the diameter of the filaments with small-diameter filaments providing much better longitudinally oriented growth. *In vivo,* microfilament-containing silicone conduits resulted in consistent axonal growth across large nerve defects (18-mm lesion gaps in rat sciatic nerve injury) at 10 weeks postinjury.[59] In the study reported here, we evaluated the ability of highly permeable and degradable polymer conduits made from poly(L-lactide-*co*-DL-lactide, 75:25) (PLA), either with or without inner microfilaments, to induce axonal migration and maturation. The results showed that combination of PLA conduits with filament scaffold enhanced axonal growth and maturation above that of either factor alone.

MATERIALS AND METHODS

PLLA filament fabrication

PLLA microfilaments were fabricated by a wet-spinning process.[59] Briefly, 10% PLLA polymer solution in chloroform (MW, 200kD, pellts from Polysciences, Inc.) with 4% BSA suspended was loaded into a glass syringe and placed in a variable speed syringe pump. TYGON® tubing connected the syringe to a Luer adapter, which was attached to an 18-guage dispensing tip that was immersed in coagulation bath. The polymer solution was pumped into the coagulation bath, which contained isopropyl alcohol. The fiber, which formed immediately as it left the dispensing tip, was then attached to a traversing roller mounted on a lathe. The roller then pulled the fiber through the bath to a final diameter of 60–80 μm. The finished fiber was air dried and stored in a desiccator until use.

Manufacture of polymer conduits

Polymer conduits were manufactured by dipping-leaching techniques. Briefly, 0.8-g purified polymer pellets [poly(L-LA-*co*-DL-LA) (75:25) (PLA)], inherent viscosity: 1.66

dL/g in $CHCl_3$ at 30°C, Birmingham Polymers, Inc., Birmingham, AL) with 1.0 mL triethyl citrate (Aldrich Chemical Company, Milwaukee, WI) and 0.8 g glucose powder (10–25 μm) (Fischer, Houston, TX) were dissolved and homogenized in 10 mL methylene chloride. Stainless steel wires coated with Polytef sheaths of 1.5 mm outer diameter were used as molds for tube dipping. The molds were systematically placed into and removed from the suspension at a constant rate for a total five dips. Every mold incubation time in the suspension was 12 s. Every solvent evaporation time was 40 s. Layers surrounding the mold became the polymer tube, which was further air dried for 2 days. Then the tube was taken off the mold, vacuumed for 1 day, and stored in a desiccator at 4°C until use. Final thickness of the polymer tubes was approximately 100 μm. Silicone tubes (1.75 mm o.d., 1.5 mm i.d.) were purchased from A-M System (Carlsborg, WA) and were autoclaved.

Scanning electron microscopy photographs

The morphologies of the prepared PLA conduits were inspected using HITACHI S-3200N Scanning Electron Microscopy (Japan). For this purpose, conduits were washed with distilled water to leach out glucose, freeze-dried, frozen in liquid nitrogen, and fractured to obtain surface- and cross-sections. The samples were gold coated using a sputter coater (EMSCOPE SC400, Japan), and the microscope was operated at 5 kV.

Final preparation and sterilization of implants

PLLA microfilaments were incubated in 70% alcohol for 1 h and dried in a sterile tissue culture hood. Silicone or PLA conduits were washed three times in 0.1 *M* phosphate buffer (PB), sterilized by incubation in 10× penicillin/streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA) in PB for 2 h at room temperature, and washed three more times in 0.1 *M* PB. For PLLA microfilament-containing implants, 16 microfilaments were bundled within either PLA or silicone tubes. Basically, the filaments were sown into the tubes. The tubes were placed between two anchored sewing needles at a distance so that four to five implants can be made in one bundling. Filaments were then sown through the tubes and looped back through the supporting needles' eye. This process was continued until the appropriate number of filaments was sown into the lumen of the tubes. The sowing process also started at the bottom of the lumen and built up toward the top. This maintained straight filament alignment within the tubes [Fig. 1(j)]. When finished, each tube was filled with matrigel and the filaments cut and trimmed. Finally, the implants were filled with Growth Factor Reduced Matrigel™ matrix (BD Biosciences, Bedford, MA) at 1:1.5 ratios in N_2 medium (Gibco, Invitrogen Corp., Carlsbad, CA). All the procedures were performed in a sterile tissue culture hood.

Surgical procedure

Adult female Sprague-Dawley rats were anesthetized by intraperitoneal injection of ketamine (66 mg/mL)/xylazine

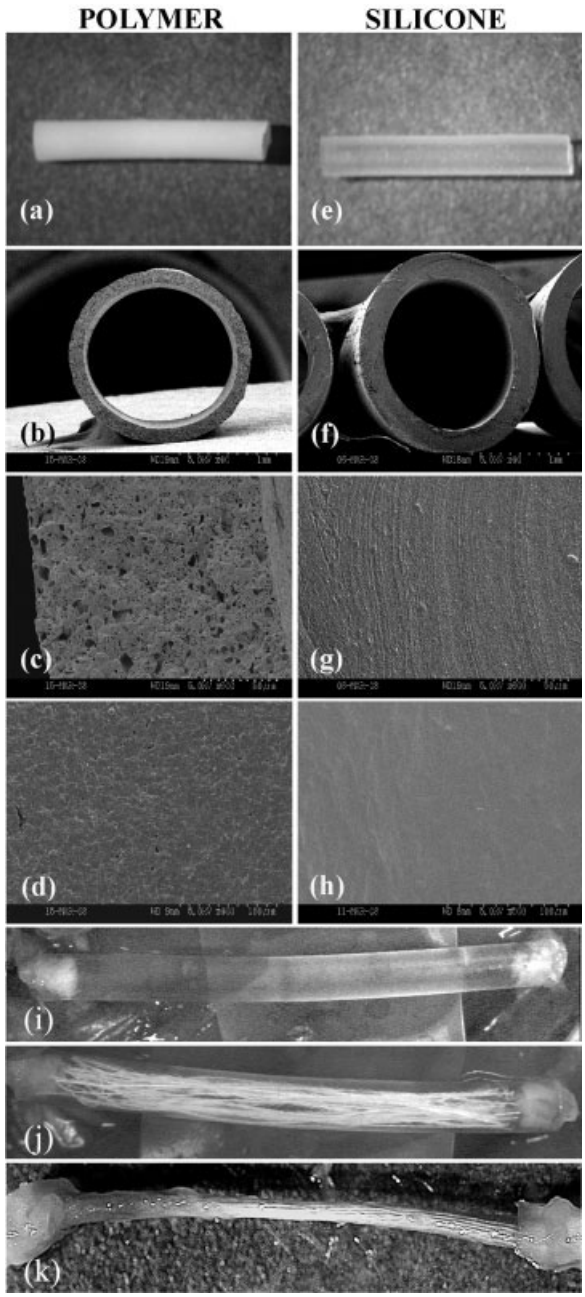

Figure 1. Microphotographs of implants made of PLA (left column) and silicone (right column). (a, e) Macroscopic views. (b, f) Cross-section images at 40× magnification (scale bar: 1 mm). The wall thickness of the PLA conduit is as consistent as that of the silicone one, and is approximately 100 μm. (c, g) High magnification (600×) images of cross sections, where the porous structure of the PLA conduit and dense, nonporous structure of the silicone conduit are illustrated (scale bar: 50 μm). The inner surface of PLA conduit (d) is even and smooth even after glucose leaching, while silicone conduit showed flatten sheet-like inner surface (h). Transparent silicone conduits represent the empty (i) and filament (j) containing conduits implanted into the 18 mm gap in the rat sciatic nerve. Cut ends of sciatic nerve extend 1.0 mm into the ends of the 2.0 cm-long tubes. Ten weeks after implantation, some filament-containing implants show nerve cable extending better nerve stumps (k). scale bar: 100 μm.

(6.66 mg/mL) at 0.1 mL/100 g. The sciatic nerve was exposed through a posterior thigh muscle-splitting incision. Sciatic nerve (7 or 9 mm) was resected to get a 14- or 18-mm nerve lesion gap. Implants were used to bridge the nerve lesions by inserting 1 mm of the proximal nerve stump and 1 mm of the distal nerve stump into either end of the conduit [Fig. 1(i,j)]. The nerve stumps were secured inside the tube with Manco Loctite QuickTite Super Glue. Ethicon and Autoclip® wound clips (2Biological Instruments, Besozzo VA, Italy) were used to staple the muscle and skin together. Animals were kept for 10 weeks with full access to food and water according to the University of Kentucky Medical Center animal care policy. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed.

Implant retrieval and histological preparation

At 10 weeks postinjury, animals were perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 *M* PB, and the implants were harvested. After polymer or silicone tubes were peeled away [Fig. 1(k)], the regenerated tissues were postfixed in 4% PFA for 2 days at 4°C. To remove the PLLA microfilaments, all the regenerated tissues went through ethanol dehydration (50, 70, 85, 95, and 100%), methylene chloride dissolving, and ethanol rehydration (above ethanol solutions in reverse order) before incubation in 30% sucrose in 0.1 *M* PB at 4°C for another 2 days. Tissues were then embedded in OCT compound, frozen and stored at −80°C.

Immunohistochemistry

Transverse sections at the distal end of the regenerated tissues (1 mm to distal nerve stump) were cut on a cryostat at 15-μm thickness and collected on slides for staining. Sections were fixed in 4% PFA for 5 min and rinsed with 0.1 *M* PBS. For blocking endogenous peroxidase activity, sections were incubated in 0.3% hydrogen peroxide diluted in methanol for 10 min. Sections were then blocked in 5% normal goat serum in PBS for 1 h, and incubated with the primary antibody overnight at room temperature. Visualization was achieved by tissue incubation in biotin-labeled secondary antibody. Biotin-labeled tissues were further processed with the Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA) and developed with diaminobenzene (DAB).

Antibodies

Rabbit anticalcitonin gene related peptide (CGRP) (1:20,000, Sigma) and mouse anti-RMDO95 (1:1000, a kind gift from Dr. Virgina Lee) were used to stain primary nociceptive afferents and phosphorylated neurofilaments (200 kDa) of axons. Rabbit anti-S100 (DAKO) (1:1200) was used to stain Schwann cells. Rabbit polyclonal anti-P0 (1:3000) for myelin labeling was a kind gift from Dr. Marie Filbin (Hunter College).

Image analysis

All stained tissue sections were viewed with a Nikon E800 light microscope at 100−400× magnification. Images were captured with Metamorph Imaging Software 5.0 (Universal Imaging Corp., Downingtown, PA). The whole transverse area, staining area of Schwann cells, and the number of CGRP- or RMDO95-positive axons at the distal end of regenerated tissues were automatically measured and counted by Metamorph Imaging Software. The number of myelinated axons was manually counted at 400× magnification with a grid-containing eye object to prevent overcounting or undercounting. Therefore, the reported number truly represented the myelinated axons in each distal cross-section.

Experimental design and statistical analysis

Factorial design was applied to improve statistical efficiency. One factor was the conduit material: silicone versus polymer. Another factor was inner filaments: empty versus 16 filaments. The continuous response variables, such as S-100 and whole tissue area, were analyzed with two-way ANOVA.[60,61] For the count data, such as CGRP and RMDO95-positive axon number as well as P0-positive myelin number, rank sum-based nonparametric Kruskal-Wallis test or generalized linear model with Poisson distribution was used.[62] If a statistically significant interaction effect existed, then comparisons were made among the levels of one factor in reference to the levels of the other factor, that is, among the cells. Otherwise, main effects of factors were then examined. p-Value = 0.05 was usually considered statistically significant, and values between 0.05 and 0.10 was considered as suggesting a trend, although statistically nonsignificant. The Fisher's protected Least-Significant Difference (LSD) correction was used for multiple *post hoc* pairwise comparisons to maintain a reasonable balance between family-wise false positive rate control and adequate power. All analyses were done using the statistical software SAS® version 9.1.

RESULTS

Parameters of polymer conduits

Internal diameters for both PLA and silicone conduits were 1.5 mm. Both proximal and distal sciatic nerve stumps were telescoped into the conduits in either 14- or 18-mm gap lesions. The thickness of PLA conduits manufactured from dipping-leaching technique was about 100 μm, as shown in scanning electron microscope (SEM) images [Fig. 1(b)]. The thick-

TABLE I
Experiment Design and Tissue Cable Formation in 14 mm and 18 mm Lesion Gaps

Group Name	Outer Guidance Channel	Inner Guidance	n1 (Number of Surgery Animals)	n2 (Number of Animals with Regenerated-Tissue across the Gap)	n2/n1 *100%
Nerve lesion gap = 14mm					
P.16F.14	PLA	16 PLLA filaments	10	10	100
P.NF.14	PLA	no PLLA filament	11	10	91
S.16F.14	silicone	16 PLLA filaments	5	5	100
S.NF.14	silicone	no PLLA filament	8	6	75
Nerve lesion gap = 18mm					
P.16F.18	PLA	16 PLLA filaments	10	10	100
P.NF.18	PLA	no PLLA filament	9	5	56
S.16F.18	silicone	16 PLLA filaments	5	5	100
S.NF.18	silicone	no PLLA filament	6	4	67

Note: P, PLA conduit; S, silicone conduit; 16F, 16 filaments bundled inside; NF, no filament bundled inside; 14 and 18, gap length. All the implants were filled with Growth Factor Reduced Matrigel™ matrix diluted with N2 medium at 1:1.5 ratios. nl: total number of animals with implantation surgery in each group; n2: total number of animals with connective tissue cable formation across the lesion gap in each group. Animal survival period: 10 weeks.

ness of polymer conduit had similar consistency as that of silicone ones [Fig. 1(b,f)]. During PLA conduit manufacture, triethyl citrate was used as a plasticizer that allows polymer conduits to easily be removed from the molds. More importantly, the plasticizer made the polymer conduits flexible enough to be bent up to 45 degrees; therefore, cracks would not occur during filament bundling or implantation. Pore size of PLA tubes were shown in cross-section SEM image with macropore size at around 10 μm and micropore size around one micron [Fig. 1(c)]. The inner surface SEM image of PLA conduit showed smooth but porous morphology [Fig. 1(d)]. Correspondingly, silicone conduits had dense nonporous walls [Fig. 1(g)] and a flattened sheet-like inner surface [Fig. 1(h)].

Tissue cable formation

Sixty-four adult female Sprague-Dawley rats were used for sciatic nerve lesion surgery: 34 for 14-mm gap and 30 for 18-mm gap. For both gap lengths, the rats were further divided into four implantation groups: PLA conduits containing 16 filaments; PLA conduits with no filaments; silicone conduits containing 16 filaments; and silicone conduits with no filaments. The number of animals used for each group is listed in Table I.

At 10 weeks postinjury, all animals receiving implants containing filaments had tissue cables formed across either 14- or 18-mm lesion gaps (Table I). Animals implanted with conduits lacking filaments had a reduced success rate ($p = 0.077$) for tissue cable formation to 91% with PLA conduits and 75% with silicone conduits for 14-mm lesion gaps. Nerve cable formation across 18-mm gaps were further reduced ($p < 0.0001$) to 56% with PLA conduits and 67% with silicone conduits. All cable formations were healthy nerve-like tissue, along which blood vessels could often be observed (images not shown). With a smooth inner surface (as shown in Fig. 1), neither PLA nor silicone conduits induce formation of a loose connective tissue stroma, a phenomenon that is often linked with fewer regenerated axons.[63] In cases were tissue cables failed to form, brown fluid filled the lumen, sometimes containing small aborted tissue cables.

The whole tissue area of distal cross-sections, as calculated using Metamorph imaging analysis software, gave a more detailed analysis about the size of regenerated tissue cables in each group (Table II). In a 14-mm lesion gap, increased cable size was apparent in groups implanted with PLA conduits ($p = 0.062$) or with filament scaffolds ($p = 0.077$) when compared with the groups implanted with silicone conduits or without filament scaffolds. With 18-mm gaps, the enhancement remained for filament scaffolds ($p = 0.073$) but not PLA conduits. These results demonstrated both nutritive support from PLA conduits and structural support from filament scaffold are important for tissue cable formation across the lesion gaps; however, the latter is more crucial, especially with long lesion gaps.

Schwann cell migration into implants

To examine if the tube material and/or filament scaffold affected Schwann cell migration, tissue sections were stained for S100 and the area occupied by Schwann cells was quantified (Table II). Two-way ANOVA showed that the filament scaffold enhanced Schwann cell migration into the conduits. This effect became especially significant in longer lesion gaps

TABLE II
Whole Tissue Area and Schwann Cell Area at the Distal End of Regenerated Tissue Cable in 14 mm and 18 mm Lesion Gaps

14mm	P.16F.14	P.NF.14	S.16F.14	S.NF.14
whole tissue area (μm^2)	655893 ± 94702	480911 ± 133929	492050 ± 90296	250056 ± 105880
Schwann cell area (μm^2)	7101 ± 1671	4384 ± 1594	4413 ± 2364	1371 ± 1869
18mm	P.16F.18	P.NF.18	S.16F.18	S.NF.18
whole tissue area (μm^2)	740257 ± 120115	397601 ± 126613	511556 ± 169869	313766 ± 155068
Schwann cell area (μm^2)	4944 ± 1285	2353 ± 1212	1197 ± 1625	1803 ± 1625

Note: P, PLA conduit; S, silicone conduit; 16F, 16 filaments bundled inside; NF, no filament bundled inside; 14 and 18, gap length. All the implants were filled with Growth Factor Reduced Matrigel™ matrix diluted with N2 medium at 1:1.5 ratios. Data are shown as mean ± SEM. Animal survival period: 10 weeks.

($p = 0.103$ in 14-mm gaps and $p = 0.030$ in 18-mm gaps). Similar results were observed for PLA conduits ($p = 0.132$ in 14-mm gaps and $p = 0.005$ in 18-mm gaps). The results confirmed our hypothesis that both polymer conduits and filament scaffolds play important roles in supporting Schwann cell migration, one of the most essential components for axonal elongation after long gap lesions.

Axons regeneration across the gap

Axonal regeneration was investigated by staining CGRP- and RMDO95-positive axons in cross-sections at the distal end of the regenerated tissue. Rabbit anti-CGRP antibodies were used to stain for nociceptive axons, which are the majority of unmyelinated axons; whereas, mouse anti-RMDO95 was used to

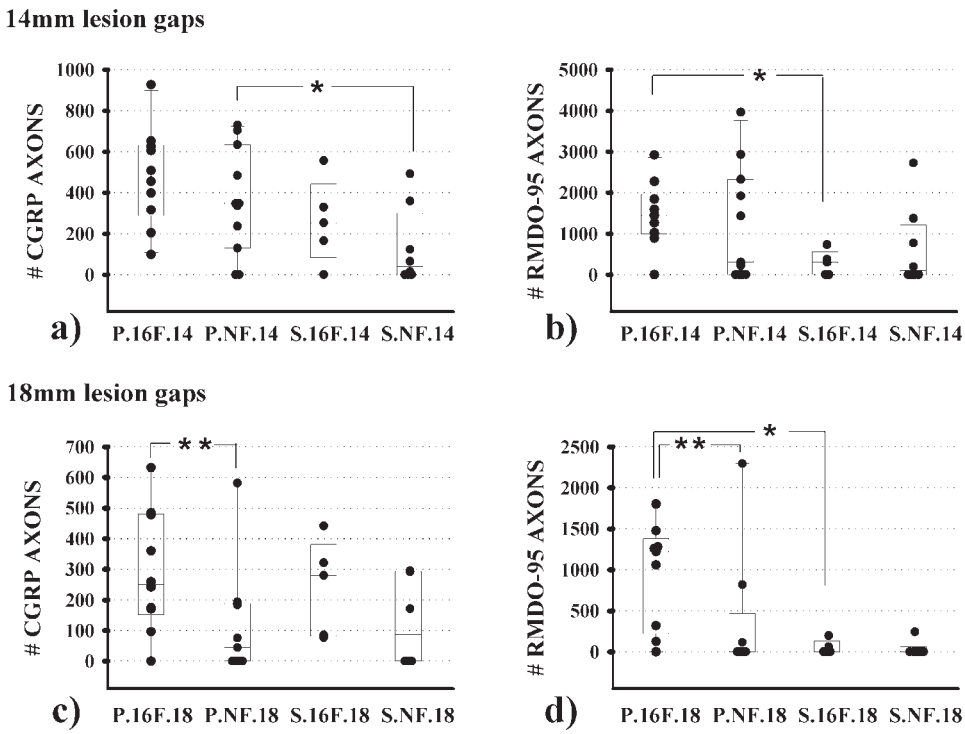

Figure 2. The number of CGRP-positive (a, c) and RMDO95-positive (b, d) axons at the distal end of regenerated tissues with various treatments in 14 mm (a, b) and 18 mm (c, d) lesion gaps. Each scatter plot list the raw data of the axon number in each group; each box plot represents a five-number summary including median of the corresponding data set. With PLA conduits, filaments (P.16F) increased unmyelinated axons, stained for CGRP, when compared to nonfilaments (P.NF), but this increment reached significance level only in 18 mm gaps. There was no statistical difference between filament (S.16F.) and nonfilament (S.NF.) groups with silicone conduits. Similar results were observed for myelinated axons stained for phosphorylated neurofilament protein (RMDO-95). On the other hand, PLA conduits significantly enhanced regeneration of unmyelinated axons compared to corresponding silicone ones in nonfilament-containing groups and in 14 mm gaps (P.NF.14 vs. S.NF.14). Increased regeneration of myelinated axons occurred in filament-containing PLA conduits (P.16F.) compared to the corresponding silicone ones (S.16F) in both 14- and 18-mm gap. However, this increment between nonfilament-containing groups failed to achieve statistical significance (P.NF. vs. S.NF.). *Reflects $p < 0.05$, and **reflects $p < 0.01$.

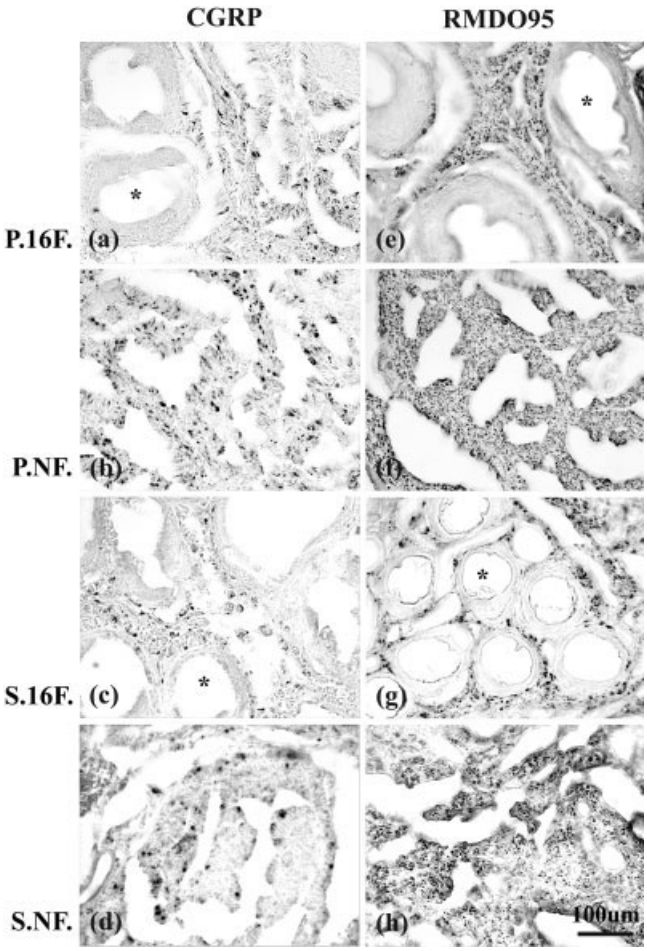

Figure 3. Photomicrographs show CGRP- (left column) and RMDO95- (right column) positive axon staining at the distal end of regenerated tissues with various implants in 14-mm lesion gaps. P, PLA conduit; S, silicone conduit; 16F, 16 filaments bundled inside; NF: no filaments bundled inside. Thin, flattened cell layers surround the PLLA microfilaments (*). Axonal migration along the filaments was consistently observed in filament-containing polymer (P.16F) or silicone conduits (S.16F) for both CGRP and RMDO95 stained images. Scale bar is 100 μm.

label phosphorylated neurofilaments, which mainly exist in myelinated axons. The immunohistochemistry staining for either anti-CGRP or anti-RMDO95 antibody was specific with very low background. As represented in Figure 3, individual axon could be clarified clearly. By threshold above the certain intensity, Metamorph Imaging Software automatically measured the total positive staining area and calculated out the axon number. If the threshold did connect some adjacent axons, a separating tool bar in the software was manually applied to separate bundled axons. In this way, our reported axon number should accurately represent the number of regenerated CGRP- and RMDO95-positive axons. The number of animals showing regeneration across lesion gaps indicated a general impression for the inductive effects of both PLA conduits and filament scaffolds on axonal regeneration (Table III). Regeneration of CGRP-positive axons in filament-containing PLA or silicone conduits was observed in almost all animals. The extent of regeneration for RMDO95-positive axons was slightly lower when compared to that of CGRP-positive axons. However, the combination of PLA conduit and filament scaffold had the highest ratios at all time points for animals with axonal regenerating toward the distal end in either 14- or 18-mm gaps.

The effect of filament scaffold on axon regeneration was further compared by the mean of distal axon number in either PLA or silicone conduits. For 14-mm lesion gaps with PLA conduits, there was no significant difference in CGRP-positive axon number between filament and nonfilament-containing implants ($p = 0.335$) [Fig. 2(a)]. However, a significant difference emerged with 18-mm gap length groups ($p = 0.009$), where the median of CGRP-positive axon number increased to 251 in filament-containing PLA conduits from 44 in corresponding nonfilament-containing ones [Fig. 2(c)]. With silicone conduits, there was no statistical difference between filament and nonfilament groups in either 14-mm ($p = 0.406$) or 18-mm lesion gaps ($p = 0.204$) [Fig. 2(a,c)]. Similar results were observed for myelinated axons expressing phos-

TABLE III
Numbers of Animals with Axons Regenerated toward the Distal End in 14 or 18 mm Gap

	CGRP	RMDO95	P0			CGRP	RMDO95	P0
14mm	n1	n2	n2	n2	18 mm	n1	n2	n2	n2
P.16F.14	10	10	9	10	P.16F.18	10	9	9	5
S.16F.14	5	4	3	2	S.16F.18	5	5	2	2
P.NF.14	11	9	7	7	P.NF.18	9	5	3	2
S.NF.14	8	5	4	4	S.NF.18	6	3	1	1

Note: P, PLA conduit; S: silicone conduit; 16F, 16 filaments bundled inside; NF, no filament bundled inside; 14 and 18, gap length. All the implants were filled with Growth Factor Reduced Matrigel™ matrix diluted with N2 medium at 1:1.5 ratios. n1: total number of animals with implantation surgery in each group; n2, total number of animals with specific axon regenerated at the distal end. Animals that failed to form connective tissue cable were considered. Samples with no distal axon staining were counted as zero for n2. Animal survival period: 10 weeks.

phorylated neurofilament protein (RMDO-95) [Fig. 2(b,d)]. In both CGRP [Fig. 3(a–d)] and RMDO95 [Fig. 3(e–h)] stained images, axonal regeneration along the filament scaffold was consistently observed through polymer or silicone conduits as we have seen in previous studies.[58,59,64] Fibroblast-like cells and macrophages were observed surrounding the microfilaments, representing a normal foreign body reaction to an inert (nonimmunogenic) substance. CGRP or RMDO95 staining for 18-mm lesion gaps appeared similar to those in 14-mm gap groups, except for a lower axon density (images not shown).

The effect of tube character on axon regeneration was also evaluated by counting the number of axons within the distal part of the implant (Fig. 2). In general, PLA polymer conduits induced greater distal axon growth when compared to either filament- and nonfilament-containing silicone conduits. However, this effect was more significant on RMDO95-positive axons than CGRP-positive axons. PLA conduits only showed a significant effect on distal CGRP-positive axon number with comparisons between nonfilament-containing PLA and silicone groups in 14-mm gaps ($p = 0.059$) [Fig. 2(a,c)]. Whereas for RMDO95-positive axons, filament-containing PLA conduits supported robust axonal regeneration when compared to the corresponding silicone ones across both 14-mm ($p = 0.042$) and 18-mm lesion gap groups ($p = 0.023$) [Fig. 2(b,d)]. The median of the distal myelinated axon number in filament-containing PLA conduits was 1443 with 14-mm lesion gaps and 1223 with 18-mm gaps. For filament-containing silicone conduits, the median decreased to 297 with 14-mm gaps and zero with 18-mm gaps. However, similar comparisons in axon numbers in nonfilament-containing groups failed to reach statistical significance ($p = 0.299$ with 14 mm, $p = 0.844$ with 18 mm) [Fig. 2(b,d)].

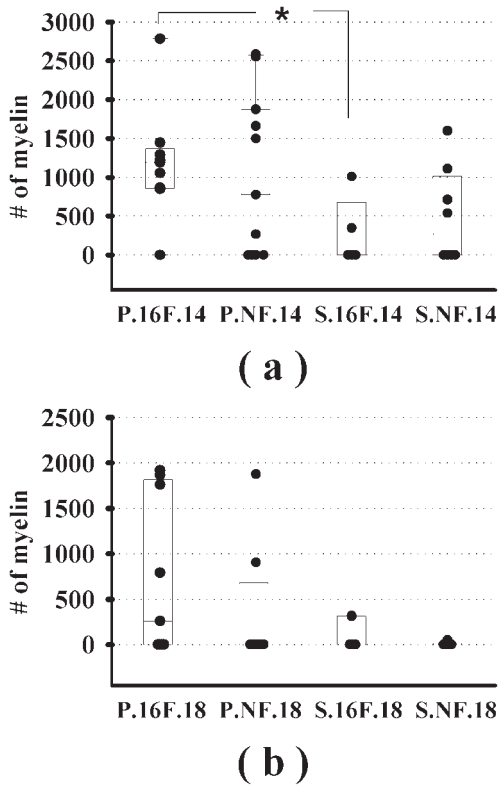

Figure 4. The number of P0-positive myelin at the distal end of regenerated tissues with various implants in 14 mm (a) and 18 mm (b) lesion gaps. Each scatter plot lists the raw data of the axon number in each group; each box plot represents a five-number summary including median of the corresponding data set. For both gap lengths, there was no significant difference in the axon number between filament (16F.) and nonfilament (NF.) containing groups in either PLA (P) or silicone (S) conduits. PLA conduits showed greater myelin number when compared to the corresponding silicone groups at both lesion lengths. Although some of the observed differences did not reach significance, a significant difference was observed between polymer and silicone conduits containing filament scaffolds at the 14-mm lesion gap (*, P.16F.14 vs. S.16F.14, $p < 0.05$).

Myelination during nerve reconstruction

Ten weeks postinjury, the extent of myelination was examined at the distal end of the nerve cables using anti-P0 antibody. With 14-mm lesion gaps, all animals in the filament-containing PLA conduit group showed P0-positive myelin profiles at the distal end of tissue cables; whereas, only 40–50% of the implants in the other groups showed myelin profiles (Table III). At the longer gap length of 18 mm, implants displaying P0-positive myelin profiles was reduced to 50% in filament-containing PLA conduits. The success rate for myelin profile further dropped to 20% in nonfilament-containing groups.

To further evaluate the effect of conduit type on myelination, we analyzed the number of P0-positive myelin for each group (Fig. 4). For either 14- or 18-mm gap lengths, there was no significant difference in the number of myelinated axons between the filament and nonfilament containing groups within either PLA or silicone conduit groups (Fig. 4). However, PLA conduits showed greater myelin number when compared to the corresponding silicone groups at both lesion lengths ($P = 0.022$ at 14 mm; $P = 0.289$ at 18 mm). Although some of the observed differences did not reach significance, a significant difference was observed between polymer and silicone conduits containing filament scaffolds at the 14-mm lesion gap ($p = 0.041$). The representative P0 stained images for each group are shown in Figure 5. Interestingly, P0 staining in PLA conduits, when compared to silicone conduits, revealed mature myelin with denser loops on larger diameter axons in both 14 and 18-mm lesion gaps [higher magnification in Fig. 5(a,b,e,f)].

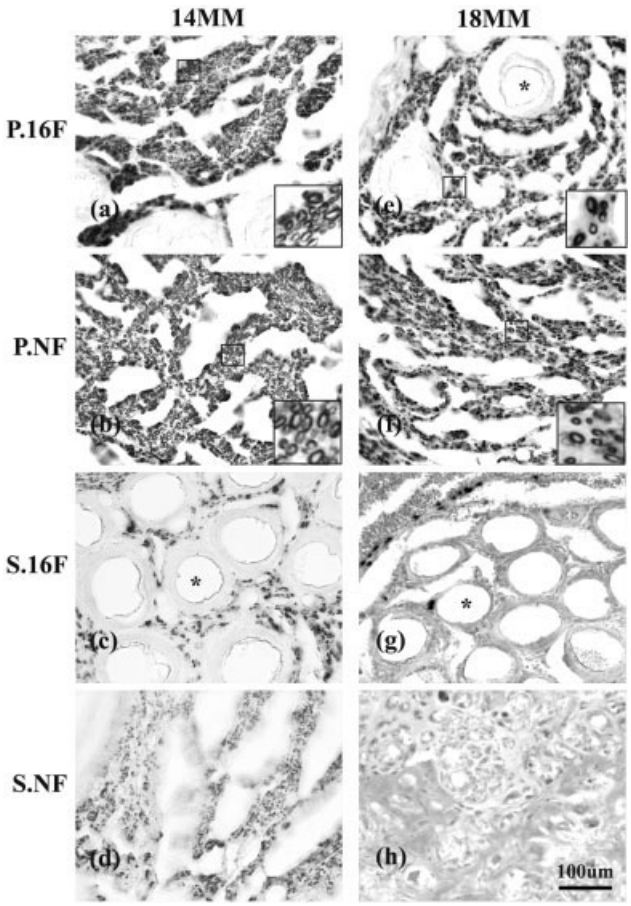

Figure 5. Photomicrographs show P0 staining for myelin of regenerating axons with various conduits in 14 mm (left column) and 18 mm (right column) lesion gaps. Thin, flattened cell layers surround the PLLA microfilaments (*). Myelinated axons migrated along the filaments in all the filament-containing conduits (polymer: a, e; and silicone: c, g). Mature myelin with denser loops on larger diameter axons appeared in PLA conduits with (a, e) or without filaments (b, f) in both lesion lengths. Scale bar is 100 μm.

DISCUSSION

Our previous studies indicate that microfilament scaffolds enhanced the cell migration and axonal guidance properties of silicone conduits for nerve repair across long gaps.[59,64] Due to their nonpermeable and nondegradable inert characters, silicone conduits provided a useful model in studying the luminal environment for repair mechanisms.[65] However, biodurable tubes could give rise to ischemia and myelin degenerative phenomena because of chronic nerve compression.[18,19,66] In the current study, we combined highly permeable and degradable poly(L-lactide-*co*-DL-lactide, 75:25) (PLA) conduits with filament scaffolds for step-by-step optimization of nerve repair. The addition of microfilaments to conduits supported longitudinally oriented cell migration and axonal regeneration, and enhancing myelination during peripheral nerve repair, especially for long lesion gaps.

PLLA microfilaments were first used to form artificial inner guidance channels for neural implants in our laboratory. No matter what the gap length was, these filament scaffolds consistently enhanced endogenous repair by organizing extracellular matrix, revascularization, and Schwann cell migrating through the lumen of the tube.[59,64] We have previously examined the migration of Schwann cells from both proximal and distal stumps in detail.[64] Schwann cells migrate extensively from both stumps within the guidance channels. However, at 10 weeks postimplantation, the longitudinal organization and density of migrating Schwann cells appeared greater only in the filament-containing implants. Without filament bundles, Schwann cell cables formed discontinuously with few cells found in the center region.[64] Even in the distal regions, Schwann cell migration was very limited and disorganized when compared with those in filament-containing groups. The failure of nonneuronal cells (such as Schwann cells) to migrate across lesion gaps explained the higher frequency of nerve regeneration failure in nonfilament-containing conduits, especially in long lesion gaps, a common problem reported with other conventional entubulation repair.[65,67] Furthermore, we previously showed that well-organized Schwann cell cable formation in filament-containing implants was critical for axon organization and regeneration.[64] Neuroma formation only appeared in nonfilament-containing implants but not filament-containing ones.

Microfilaments might also aid nerve repair by providing the initiation substrate for Schwann cell migration out of nerve stumps and into the lumen of the conduit, while preventing myofibroblasts from effectively "walling off" the nerve stump. One of the chief components of the regenerating epineurium is myofibroblast, and it is been hypothesized that competitive interactions between myofibroblasts and axon growth at the nerve stump influences nerve repair. If myofibroblasts encapsulate the nerve stump axon regeneration is prevented and a neuroma forms.[68,69] We have previously shown that microfilaments provide a substrate that enhances and organizes Schwann cell migration.[58,64] Increased migration of Schwann cells may therefore cut out compete myofibroblast capsule formation and prevent sealing of the nerve stump. Because Schwann cells are a very important growth supportive substrate for axonal regeneration, their directional migration out of the nerve stump and along the microfilaments establishes a pathway for regenerating axons. This process improved structural alignment leading to better axon fasciculation and minimal neuroma formation especially in long lesion gaps.[59,64]

Compared to 32 filaments in our previous works, we used only 16 filaments in this study to reduce the filaments space occupation in the regenerated tissue

cable. But at the same time, the packing density of the guidance channels was lowered and went out of the optimal packing density window for best axon regeneration.[59] This might be an explanation for the nonsignificant difference between filament- and nonfilament-containing silicone conduits on the number of distal axons in either 14- or 18-mm gap length. To balance the supporting density and space occupation of the filament scaffold, other filaments with a faster degradation rate are being tested in our laboratory.

Artificial conduits made from biodegradable materials have been explored as a means of providing mechanical continuity across the gap but avoiding of problems associated with chronic compression in long term. As regenerating nerve fibers grow and mature across 10–18-mm gaps in the rat sciatic nerve, it is necessary to achieve a temporal balance to maintaining the structural integrity of the polymer conduit in accordance with the axonal growth rate. Polylactide (PLA), polyglycolide (PGA), and their copolymers (PLGA) are being actively investigated for use as artificial conduits. A potential problem associated with 75:25 PLGA conduits is weakness due to degradation that results in conduit collapse and inhibition of axon growth.[70,71] Although poly (L-lactide) (PLLA) conduits are more stable and showed improved nerve regeneration,[20,72] their very slow degradation rate might result in the same nerve compression as biodurable conduits. As an osteosynthesis material, PLLA was shown to remain for about 3 years after implantation.[73] To overcome these problems, we decided to use poly (L-lactide-*co*-DL-lactide, 75:25) (PLA) as a conduit material, where DL-lactide, an amorphous component, was added to lower crystalline of PLLA, and consequently increase degradation rate. At 10-weeks postinjury, PLA conduits were wrapped by a thin fibroblast capsule indicating a normal foreign body reaction.[59] The polymer conduits was not as strong as those prior to implantation, but still maintained tube integrity without swelling or collapsing. At the completion of these studies, we observed no collapsed tubes. We estimated reabsorption of our PLA conduits *in vivo* would take about 1 year to complete, although degradation tests were not done in this study.

According to the method of Rodriguez and Navarro et al.,[74] after water extraction of 10 μm particles of glucose powder suspended in the wall of poly (lactide-*co*-caprolactone) conduits would generate pores with a molecular weight cutoff greater than 2000 kDa. Such pores would allow the diffusion of extraneural wound-healing factors to immigrate through the tube wall, as well as fibroblast and reticuloendothelial cells that might aid revascularization and extracellular matrix formation important in the early stages of nerve repair.[75,76] Our results were in agreement with these observations, in which PLA conduits were associated with higher levels of tissue cable formation when compared to silicone conduits. These observations extended to both filament-containing and empty PLA lumens, and more significantly at short lesion gaps.

At 10 weeks postinjury, the thickness of fibrous capsule covering PLA conduits was comparable to that around silicone conduits. The fibrous cover formed around the conduit reestablishes the perineurial–nerve barrier, and limits the exchange between the tube lumen and outer milieu. Azzam et al.[77] stated that high permeability plays a role mainly in the early stages of regeneration. However, compared to silicone conduits, the permeability and degradability of PLA conduits contributed to the significance of continuous exchange with the surrounding environment, which is of interest for a later stage migration and maturation of Schwann cells and axons. Except for the blood vessels within tissue cables in both PLA and silicone conduits, transverse blood vessels only appeared growing along the wall of PLA conduits, which could supply additional oxygen and nutritive to the regenerating tissue. These observations for continuous supply of extra nutritive factors better explains our results of increased nerve repair and maturation with PLA but not silicone conduits.

Evidence of increased axonal maturation came from distal RMDO95-positive axon counts, neurofilament phosphorylation occurs predominately in lager caliber axons during later stages of regeneration.[78,79] Combining PLA conduits and filament scaffolds increased the number of RMDO95 labeled axons but not CGRP within the distal region of the implant, when compared to corresponding silicone conduits. However, in the absence of the filament scaffold no differences were observed for the number of RMDO95-positive axons between the PLA and silicone conduits, again demonstrating the indispensable role of filaments for contact guidance during nerve regeneration. Another measure for maturation and health of the repaired nerve is the extent of remyelination, as determined using P0 staining. In the mature state the bulk of the myelin sheath is compact, so that the extracellular space between the turning loops is drastically reduced and the cytoplasm is removed to yield the intraperiod and major dense lines.[80] The number of myelinated axons within PLA conduits on average were 2.8-fold higher that those within silicone conduits. Myelin profiles in the distal region of PLA implants also showed better morphology with larger diameter and clearly compacted myelin sheath. These data suggest that permeable biodegradable PLA tubes are important for both early regenerative and later maturation of the repaired nerve. This may be due to the better revascularization, compartmental flow of nutrients, increased axonal regeneration, and reduced constriction by our PLA conduits when compared to silicone tubes.

SUMMARY AND FUTURE WORK

The principle of axonal growth and maturation during nerve repair are based upon at least three proposed groups of factors, including neurotrophism, contact guidance, and neurotropism.[17] According to this principle, by combining a filament scaffold for inner contact guidance and highly permeability as well as degradability of PLA conduits for better exchange of nutritional and neurotrophic factors, in general, demonstrated superior performance for eliciting nerve repair than nonpermeable conduits without filaments. Further improvements are presently being examined using polymer filament scaffolds as carriers of biologically active molecules. These filament-containing conduits have recently been shown to greatly enhance dorsal root repair and functional recovery after a 6mm excision of lumbar roots 4 and 5.[81]

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